Attorney's Docket No. 5051-445

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Moyer et al.
Serial No.: 09/912,072
Filed: July 24, 2001
For: IDENTIFICATION OF POINSETTIA CULTIVARS

Examiner: M. Sheinberg
Group Art Unit: 1634

Declaration of Dr. James W. Moyer under 37 C.F.R. § 1.132

I, James W. Moyer, do hereby declare and state as follows:

1. I am a Professor of Plant Pathology and Head of the Department of Plant Pathology at North Carolina State University. I received my undergraduate degree in Agronomy at Washington State University. I did my masters and doctoral work in plant pathology at The Pennsylvania State University. One of the research areas on my laboratory is a floral crop program that has focused on techniques for development of reliable fingerprinting technology as an aid to cultivar identification and more recently the development of tools for molecular marker assisted breeding.

2. I am a named inventor on U.S. Patent Application No. 09/912,072 (*hereinafter* "the '072 application").

3. I have read the following publications cited by the Examiner in connection with the '072 application:

- Ling et al., *HortScience* 32:122-124 (1997)
- Barcaccia et al., *J. Horticultural Science & Biotechnology* 74:243-250 (1999)
- Singh et al., *Crop Improv.* 25:15-20 (1998) (*referred to as "Sukhwinder et al."*)
- Barker et al., *Genome* 42:173-182 (1999)

4. Ling et al. concerns the use of RAPD techniques to compare the DNA of nine commercial poinsettia cultivars, which were from widely differing groups. Thus, the RAPD analysis of Ling et al. did not have to be robust to distinguish these cultivars. Singh et al., Barker et al., and Barcaccia et al. applied AFLPs to compare the fingerprints of cultivars from rice, willow and *Pelargonium* (geranium), respectively.

5. Prior to the work described in the '072 application, it would not have been obvious from Ling et al. that AFLP analysis of poinsettias could distinguish and estimate genetic relationships among different cultivars. AFLP analysis had been used in other plants, primarily crop plants such as rice and willow as described in the Singh et al. and Barker et al. publications. Some recent work with AFLPs had been reported in ornamental plants including *Pelargonium* as described by Barcaccia et al.

However, it simply was not known whether AFLP analysis would be able to detect sufficient inter-cultivar polymorphisms among poinsettia cultivars. Barcaccia et al.'s work with geranium would not have been predictive with respect to poinsettia, because the gene pools of these plants are distinct.

6. Poinsettia is an asexually reproducing species, with a narrow genetic base. Most poinsettia cultivars have been identified by selection of sports or induced mutations. As a result, there is very little pedigree information available for poinsettia. Prior to the invention described in the '072 application, it was uncertain whether there would be sufficient genetic polymorphisms detectable by AFLP among poinsettia cultivars.

7. Further, it was not at all obvious in advance that the polymorphisms and genetic fingerprints would be powerful enough to track the breeding history or pedigree of a broad range of poinsettia cultivars and that the different breeding families would have distinct and closely related fingerprints. One of the exciting discoveries that came out of the work described in the '072 application was the finding that differences in the AFLP fingerprints were reflective of breeding lineage. While there is speculation in the literatiure about relationships, none of the cited publications (Ling et al., Singh et al., Barker et al., Barcaccia et al.) demonstrate this relationship between polymorphism and breeding history.

8. The unpredictability in fingerprinting methods as applied to poinsettia is also evident in our work with microsatellites. We have tried to evaluate genetic relationships among poinsettia cultivars using microsatellite simple sequence repeat (SSR) analysis. Microsatellites are sections of DNA composed of repeats of short motifs (*e.g.*, CA, GTG, TGCT, *etc.*) arranged in tandem. The sequence surrounding the repeat region is usually conserved, allowing amplification primers to be designed so that the repeat region and a short flanking sequence can be amplified. Polymorphisms are observed in the number of repeats present.

9. Approximately 700,000 bases of poinsettia sequence were obtained from a genomic library constructed of partially digested 'Freedom Pink' DNA. Using a computer algorithm, the sequence was scanned for the presence of SSR motifs of significant size to be polymorphic based on previous studies (Cardle et al., *Genetics* 847-854 (2000); Alvarez et al., *Theor. Appl. Genet.* 103:1283-1292 (2001)). The minimum number of repeats selected were 6 for dinucleotide motifs, 5 for trinucleotide motifs, 4 for tetranucleotide motifs, and 4 for pentanucleotide motifs. As far as I am aware, no other studies have reported sextanucleotide motifs; the minimum number of repeats for this motif was set at 3. A total of 20 SSR motifs were isolated. They consisted of: 11 dinucleotide, 6 trinucleotide, 1 tetranucleotide, 1 pentanucleotide, and 1 sextanucleotide. Primers were designed for 18 of the 20 SSRs; two of the motifs were near the end of the cloned insert such that there was not sufficient flanking region in which to design a primer.

10. The plant material selected for evaluation consisted of 48 cultivars of poinsettia representing 12 of the major cultivar groups of color sports, as well as 4

other cultivars. These groups included Angelika, Annette Hegg, Celebrate, Cortez, Freedom, Gross, Lilo, Nutcracker, Pepride, Peterstar, Sonora, and V14 Glory. The 4 additional cultivars selected were 'Winter Rose', 'Pearl', 'Prestige', and 'Snowcap'. Duplicate samples of 2 cultivars taken from different plants were used as controls.

11. Twelve of the primer pairs amplified a fragment of the predicted size, whereas the rest could not be optimized and either did not amplify or resulted in too complex a pattern to evaluate. The primer pairs amplified from 1-5 alleles each, with an average of 2 alleles. Three primer pairs amplified one allele. Six of the loci were polymorphic, with 2 to 4 alleles. Duplicate cultivars consistently amplified the same number of alleles.

12. Statistical analysis of the data revealed a narrow range of distances and low resolution of cultivars and cultivar groups on a dendrogram (Appendix 1; attached). Shared allele distances ranged from 0-0.25. The largest distance, 0.25 was between the: Hegg group and V14 Glory Red; V14 Glory Pink/V14 Glory White and Pepride Red, Winter Rose, and the Cortez group; Winter Rose and the Lilo group; and the Sonora group and the Lilo group. Many cultivar comparisons had a distance of 0 and could not be differentiated, as seen in the attached dendrogram. Some cultivar groups could be differentiated from each other and formed unique clusters on the dendrogram; Hegg, Cortez, Sonora, and Lilo cultivar groups formed unique clusters with a distance of 0. Other cultivar groups were divided or clustered with unrelated groups. The pink and white cultivars of the Celebrate 2 and V14 Glory groups each formed unique clusters separate from the Red "parent" cultivars of these groups. The white cultivars Angelika White, Snowcap, Nutcracker White, and Pearl clustered together with a distance of 0. Finally, a large cluster with a 0 distance was made up of the Freedom, Peterstar, and Gross groups as well as the Angelika and Nutcracker groups minus the white cultivars.

13. As the results described above demonstrate, the power of microsatellite techniques to differentiate cultivars was not evident in poinsettia, likely due to the narrow genetic base of this crop resulting from the methodologies used in poinsettia breeding programs, which rely heavily on mutation breeding and selection of sports. This finding is unexpected; based on the known properties of microsatellites, I would have expected this approach to have worked as well, or even better, than AFLPs in distinguishing poinsettia cultivars (*see, e.g.*, Margante et al., *Plant J.* 3:175-182 (1993); Maguire et al., *TAG* 104:388-398 (2002). It is known that SSR markers tend to have a higher level of heterozygosity than AFLP markers due to codominance of SSR loci. In addition, SSR markers generally have greater somatic stability than AFLP markers. Finally, SSR techniques are typically found to be more technically reproducible than AFLP. However, from our data, it appears that the narrow genetic base of poinsettia lacks polymorphisms in the SSR loci.

14. RAPD, AFLP and SSR markers are each distinct. RAPD markers detect polymorphisms based on hybridization of short primers to random locations in the genome, whereas AFLP markers detect polymorphic restriction sites in the genome or at least polymorphisms in close proximity to restriction sites.

Microsatellites detect polymorphisms in the number of short tandem motif repeats that are present in the genome. RAPD, AFLP and SSR would therefore each detect a different subset of polymorphisms. The microsatellite data discussed above demonstrates that for poinsettia you must detect the "right" polymorphisms in order to distinguish among poinsettia cultivars.

15. The narrow genetic base of poinsettia and the failure of microsatellite analysis to distinguish poinsettia cultivars indicate that there is unpredictability in the application of fingerprinting techniques to poinsettia, with each approach needing to be evaluated on a case-by-case basis. It therefore would not have been obvious prior to the experimentation described in the '072 application that sufficient AFLP polymorphisms would be present in the poinsettia gene pool for AFLPs to be successful in distinguishing and determining genetic relationships among poinsettia cultivars.

16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

James W. Moyer

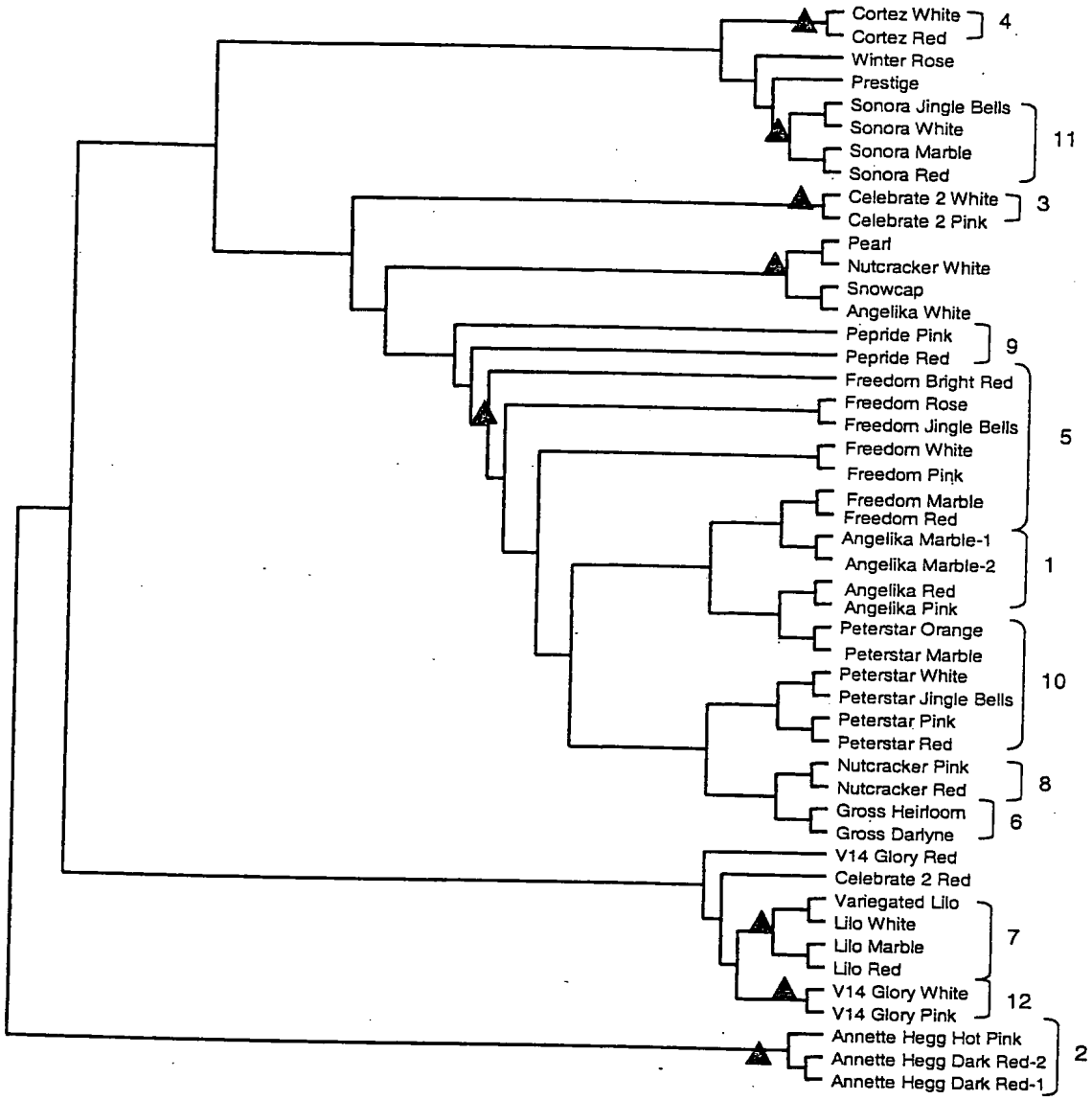
James W. Moyer, Ph.D.

5/19/05

Date

Attachment: Appendix A

APPENDIX I



Dendrogram of 44 poinsettia cultivars and 2 duplicates generated with microsatellite data using shared allele distance and Neighbor-Joining clustering. Brackets and numbers denote cultivar groups. Triangles denote clusters of cultivars with a distance of 0.

haploid [Gk. *haploos,* single]: Having only one set of chromosomes (*n*), in contrast to diploid (2*n*).

hardwood: A name commonly applied to the wood of a dicot tree.

Hardy-Weinberg law: The mathematical expression of the relationship between the relative frequencies of two or more alleles in a population; it demonstrates that the frequencies of alleles and genotypes will remain constant in a random-mating population in the absence of inbreeding, selection, or other evolutionary forces.

haustorium, *pl.* haustoria [L. *haustus,* from *haurire,* to drink, draw]: A projection of fungal hypha that functions as a penetrating and absorbing organ.

heartwood: Nonliving and commonly dark-colored wood in which no water transport occurs; it is surrounded by sapwood.

heliotropism [Gk. *helios,* sun]: *See* solar tracking.

hemicellulose (hĕm'ĭ-sĕl'u-lōs): A polysaccharide resembling cellulose but more soluble and less ordered; found particularly in cell walls.

herb [L. *herba,* grass]: A nonwoody seed plant with a relatively short-lived aerial portion.

herbaceous: An adjective referring to nonwoody plants.

herbarium: A collection of dried and pressed plant specimens.

herbivorous: Feeding upon plants.

heredity [L. *heredis,* heir]: The transmission of characteristics from parent to offspring through the gametes.

hermaphrodite [Gk. for Hermes and Aphrodite]: An organism possessing both male and female reproductive organs.

hetero- [Gk. *heteros,* different]: Prefix meaning "other" or "different."

heterocyst [Gk. *heteros,* different, + *cystis,* a bag]: A transparent, thick-walled, nitrogen-fixing cell that forms in the filaments of certain cyanobacteria.

heteroecious (hĕt'er-ē'shŭs) [Gk. *heteros,* different, + *oikos,* house]: As in some rust fungi, requiring two different host species to complete the life cycle.

heterogamy [Gk. *heteros,* other, + *gamos,* union or reproduction]: Reproduction involving two types of gametes.

heterokaryotic [Gk. *heteros,* other, + *karyon,* kernel]: In fungi, having two or more genetically distinct types of nuclei within the same mycelium.

heteromorphic [Gk. *heteros,* different, + *morphe,* form]: A term used to describe a life history in which the haploid and diploid generations are dissimilar in form.

heterosis [Gk. *heterosis,* alteration]: Hybrid vigor, the superiority of the hybrid over either parent in any measurable character.

heterosporous: Having two kinds of spores, designated as microspores and megaspores.

heterothallic [Gk. *heteros,* different, + *thallus,* sprout]: A term used to describe a species, the haploid individuals of which are self-sterile or self-incompatible; two compatible strains or individuals are required for sexual reproduction to take place.

heterotroph [Gk. *heteros,* other, + *trophos,* feeder]: An organism that cannot manufacture organic compounds and so must feed on organic materials that have originated in other plants and animals; *see also* autotroph.

heterozygous: Having two different alleles at the same locus on homologous chromosomes.

Hill reaction: The oxygen evolution and photoreduction of an artificial electron acceptor by a chloroplast preparation in the absence c carbon dioxide.

hilum [L. *hilum,* a trifle]: (1) Scar left on seed after separation of see from funiculus; (2) the part of a starch grain around which the starc is laid down in more or less concentric layers.

histone: The group of five basic proteins associated with the chromo somes of all eukaryotic cells.

holdfast: (1) Basal part of a multicellular alga that attaches it to a solid object; may be unicellular or composed of a mass of tissue; (2 cuplike structures at the tips of some tendrils, by means of whicl they become attached.

homeo-, homo- [Gk. *homos,* same, similar]: Prefix meaning "similar" or "same."

homeostasis (hō'me-ō-stā'sĭs) [Gk. *homos,* similar, + *stasis,* standing] The maintaining of a relatively stable internal physiological environment within an organism, or a steady-state equilibrium in a population or ecosystem. Homeostasis usually involves feedback mechanisms.

homokaryotic [Gk. *homos,* same, + *karyon,* kernel]: In fungi, having nuclei with the same genetic makeup within a mycelium.

homologous chromosomes: Chromosomes that associate in pairs in the first stage of meiosis; each member of the pair is derived from a different parent. Homologous chromosomes are also called homologues.

homology [Gk. *homologia,* agreement]: A condition indicative of the same phylogenetic, or evolutionary, origin, but not necessarily the same in present structure and or function.

homosporous: Having only one kind of spore.

homothallic [Gk. *homos,* same, + *thallus,* sprout]: A term used to describe a species in which the individuals are self-fertile.

homozygous: Having identical alleles at the same locus on homologous chromosomes.

hormogonium, *pl.* hormogonia: A portion of a filament of a cyanobacterium that becomes detached and grows into a new filament.

hormone [Gk. *hormaein,* to excite]: A chemical substance produced usually in minute amounts in one part of an organism, from which it is transported to another part of that organism on which it has a specific effect.

host: An organism on or in which a parasite lives.

humus: Decomposing organic matter in the soil.

hyaloplasm: *See* cytoplasmic ground substance.

hybrid: Offspring of two parents that differ in one or more heritable characteristics; offspring of two different varieties or of two different species.

hybridization: The formation of offspring between unlike parents.

hybrid vigor: *See* heterosis.

hydrocarbon [Gk. *hydro,* water, + L. *carbo,* charcoal]: An organic compound that consists only of hydrogen and carbon atoms.

hydrogen bond: A weak bond between a hydrogen atom attached to one oxygen or nitrogen atom and another oxygen or nitrogen atom.

hydrolysis [Gk. *hydro,* water, + *lysis,* loosening]: Splitting of one molecule into two by addition of the H^+ and OH^- ions of water.

hydrophyte [Gk. *hydro,* water, + *phyton,* a plant]: A plant that depends on an abundant supply of moisture or that grows wholly or partly submerged in water.

hydroxyl group: An OH^- group; a negatively charged ion formed by the dissassociation of a water molecule.

Raven, et.al., Biology of Plants, Worth Publ. NY, NY pp. 791 (1992)

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Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs

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Abstract DNA-based fingerprinting technologies have proven useful in genetic similarity studies. RFLP is still most commonly used in the estimation of genetic diversity in plant species, but the recently developed PCR-based marker techniques, RAPDs, SSRs and AFLPs, are playing an increasingly important role in these investigations. Using a set of 33 maize inbred lines we report on a comparison of techniques to evaluate their informativeness and applicability for the study of genetic diversity. The four assays differed in the amount of polymorphism detected. The information content, measured by the expected heterozygosity and the average number of alleles, was higher for SSRs, while the lowest level of polymorphism was obtained with AFLPs. However, AFLPs were the most efficient marker system because of their capacity to reveal several bands in a single amplification. In fact, the assay efficiency index was more than ten-fold higher for AFLPs compared to the other methods. Except for RAPDs, the genetic similarity trees were highly correlated. SSR and AFLP technologies can replace RFLP marker in genetic similarity studies because of their comparable accuracy in genotyping inbred lines selected by pedigree. Bootstrap analysis revealed that, in the set of lines analysed, the number of markers used was sufficient for a reliable estimation of genetic similarity and for a meaningful comparison of marker technologies.

Key words *Zea mays* L. · Genetic relationship · Molecular markers · DNA-fingerprinting · Genetic diversity

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Introduction

Knowledge of germplasm diversity and of relationships among elite breeding materials has a significant impact on the improvement of crop plants (Hallauer et al. 1988). In maize, this information is useful in planning crosses for hybrid and line development, in assigning lines to heterotic groups, and in plant variety protection. It can be obtained from pedigree and heterosis data, from morphological traits or using molecular markers which detect variation at the DNA sequence level (Smith and Smith 1992). In particular, DNA-based polymorphisms are a powerful tool in the assessment of the genetic similarity between breeding stocks (reviewed in Lee 1995).

The discrimination power of restriction fragment length polymorphisms (RFLPs) has been extensively studied in maize, as has their use in establishing relationships with yield and heterosis (Melchinger 1993). However, there are several drawbacks to RFLPs that have stimulated the development of alternative marker systems: large quantities of DNA are in fact required for RFLP analysis, which is costly, and the technique is difficult to automate. Moreover, it requires sizeable laboratories and specialised equipment.

Various PCR-based marker techniques have recently been successfully introduced in the fingerprinting of plant genomes (Welsh and McClelland 1990; Kesseli

et al. 1994) and in genetic diversity studies (Tinker et al. 1993). Among them, random amplified polymorphic DNA (RAPD) analysis is quick (Welsh and McClelland 1990; Williams et al. 1990) and well adapted for the efficient non-radioactive DNA fingerprinting of genotypes (dos Santos et al. 1994; Thormann et al. 1994). Problems with the reproducibility of amplification and with the scoring of error data have been reported for RAPDs (Demeke et al. 1997; Karp et al. 1997).

Eukaryotic genomes are interspersed with tandem repeats of DNA, referred to as microsatellites or simple sequence repeats (SSRs). SSR polymorphisms have been extensively used as genetic markers in mammals (Tautz 1989); they occur frequently also in plant genomes, showing an extensive variation in different individuals and accessions (Akkaya et al. 1992; Senior and Heun 1993; Wu and Tanksley 1993). SSR loci are co-dominant markers more informative than RAPDs and RFLPs (Russell et al. 1997). Specific technical developments are underway (Mitchell et al. 1997) that should result in the provision of SSRs that will be faster, more standardised and more effective than RFLP technology.

Amplified fragment length polymorphism (AFLP™) is a multilocus marker technique developed by Vos et al. (1995). AFLP markers are genomic fragments detected after selective PCR amplification which provide a number of appealing features in the fingerprinting of genomes of different complexity, including that of maize (Vos et al. 1995). The AFLP technique has been used to identify markers linked to disease resistance loci (Becker et al. 1995; Cervera et al. 1996), to fingerprint DNAs (Vos et al. 1995; Sharma et al. 1996), and to assess relationships between molecular polymorphism and hybrid performance in maize (Ajmone-Marsan et al. 1998).

A comparison of different marker techniques is timely, even though the utility of different molecular markers for soybean and barley germplasm has already been reported (Powell et al. 1996; Russell et al. 1997). The objectives of the present study were: (1) to compare the informativeness of different molecular markers and their applicability for genetic diversity analysis, genotype identification and variety protection purposes, (2) to determine the genetic similarity obtained with RFLP- and PCR-based techniques in a set of maize inbred lines, and (3) to compare their effectiveness in estimating genetic similarity among maize inbreds.

Materials and methods

Plant materials and DNA extraction

Thirty three inbred lines were chosen to explore the diversity of maize germplasm. All these inbreds have been extensively used in the production of hybrid seed and in maize breeding programs. Pedigree information was previously described in Livini et al. (1992). Based on available information and on the heterotic behaviour in crosses, 13 (A641, B14 A, B37, B73, B84, Cm109 Lo1016, Lo916, Lo950, Lo951, Lo964, Lo999, and N28) can be associated with the Iowa Stiff Stalk Synthetic (BSSS) heterotic group, 13 (A619, C103, C123, H99, Lo881, Lo924, Lo976, Lo1077, Mo17, Oh43, Va22, Va59, and Va85) with the Lancaster Sure Crop (LSC), two to Wf9 (WF9 and Pa91), three to W153R (W153R, Lo932, and Lo944), and two to HY (H55 and H96). Genomic DNA was isolated from a bulk of 20–30 shoots of 7–9-day old germinated seedlings and extracted using the CTAB method as previously described (Livini et al. 1992).

Nucleic-acid manipulation and molecular-marker assays

Conditions for restriction enzyme digestion, gel electrophoresis for RFLP, Southern transfer hybridisation, and autoradiography followed Livini et al. (1992). Forty seven genomic clones from the UMC and BNL collections and two restriction enzymes (*Eco*RI and *Hin*dIII) were used to characterise 53 RFLP loci in the 33 inbred lines. A total of 253 RFLP bands were binary coded as 1 or 0 for the presence or absence of such loci in each line, respectively.

RAPD amplification was performed as described by Ajmone-Marsan et al. (1993) using a Perkin Elmer 9600 Thermal Cycler. Reaction products were analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. A total of 85 primers (Operon Technologies, California, USA) were surveyed in two inbred lines. Twenty five primers showing reproducible, and clearly scorable, polymorphic (present or absent) fragments, were used to fingerprint the 33 inbreds.

The primers for the SSR markers were synthesised according to the sequences published by Senior and Heun (1993) and Taramino and Tingey (1996). SSR procedures were those described by Taramino and Tingey (1996). Processed fragments, along with loading dye and internal size standards, were run out on a 6% acrylamide gel (Pfeiffer et al. 1997) using an Automated Laser Fluorescent sequencing electrophoresis unit (Pharmacia). Fragments were labelled with fluorescein by direct incorporation of F-12-dUTP (2-μM final concentration) during the PCR reaction. Un-incorporated labelled nucleotide was removed by ethanol precipitation prior to loading samples on the gel. Data were processed using Fragment Manager Software v. 1.1 (Pharmacia). SSR bands were sized first and then binary coded by 1 or 0 for their presence or absence in each line.

AFLP marker analysis was according to Vos et al. (1995). Briefly, total genomic DNA (400 ng) was restricted with 5 U of *Eco*RI (rare cutter) and 5 U of *Mse*I (frequent cutter) (Pharmacia), and double-stranded adapters ligated to the fragment ends. The structure of the adapter sequences, pre-amplification, amplification and polyacrylamide-gel electrophoresis conditions were as in Ajmone-Marsan et al. (1998). Polymorphic amplification products were visualised by autoradiography and scored manually. All AFLP polymorphisms were scored as dominant markers.

All names of the RFLP probes and the nucleotide sequences of the primers used for the amplification of AFLP, RAPD, and SSR markers are available on request.

Data analysis

The average number of alleles per locus, the allele frequency, the expected heterozygosity (He), and the effective number of alleles per locus were calculated as reported by Morgante et al. (1994). The total number of effective alleles (Ne) surveyed by RFLP, RAPD, SSR, and AFLP analyses was calculated by summing the number of effective alleles of all the analysed loci as $Ne = \sum ne\,(i)$. To compare the efficiency among the four methods, where RFLPs and SSRs generally detect multiple alleles and one band per assay, whereas RAPDs and AFLPs detect two alleles and multiple bands per assay,

an assay efficiency index (Ai) was calculated. Ai combines the effective number of alleles identified per locus and the number of the polymorphic bands detected in each assay as Ai = Ne/P, where Ne is the total number of effective alleles detected and P is the total number of assays performed for their detection.

The genetic similarities (GSs) from RFLP, RAPD, SSR, and AFLP data were calculated among all possible pairs of lines using the Dice similarity index as in Nei and Li (1979). The co-ancestry coefficient, *f*, between lines related by pedigree, was calculated as previously reported (Ajmone-Marsan et al. 1992). Cluster analyses were based on similarity matrices obtained with the unweighted pair group method using arithmetic averages (UPGMA) (Rohlf 1990) and relationships between inbred lines were visualised as dendrograms. For each dendrogram the co-phenetic coefficients between the matrix of genetic similarities and the matrix of co-phenetic values were computed using appropriate routines of the NTSYS-pc package. The significance of the co-phenetic correlation observed was tested using the Mantel matrix correspondence test (Mantel 1967).

The bootstrap procedure was employed to determine the sampling variance of the genetic similarities calculated from the data sets obtained with the different marker systems. All data, irrespective of the dual or multiallelic nature of the marker system, were scored in the form of a binary matrix. For each pair of inbreds, the Dice similarity index (GS) was calculated from the 2000 random subsamples at different sample sizes (10, 50, 100, 150, 200, and all bands when the total exceeded 200). Bootstrap standard deviation estimates were based on 2000 samples. The calculations were performed with the SAS macro "BOOT" (Jackknife and Bootstrap Analyses, SAS Institute Inc.).

Results

Levels of polymorphisms

The 33 inbred lines were surveyed with the four different marker systems. All of the molecular markers were able to uniquely fingerprint each of the inbred lines. The levels of polymorphism detected with each marker system and the index comparing their informativeness are reported in Table 1. The total number of assays ranged from only six primer combinations for AFLPs to 53 probe/enzyme combinations for RFLPs. The total number of polymorphic bands identified ranged from 90 for RAPDs to 253 for RFLPs. An average number of 4.8 alleles per locus, with an average effective number of 3.2 alleles per locus, ranging from 1.2 to 6.5, could be distinguished for each probe/enzyme combination using RFLPs. This value increased to 6.8 with SSRs, with an average number of effective alleles of 4.4 per locus, ranging from 1.1 to 6.6, while for RAPDs and AFLPs these values were lower (1.6 for both). This was reflected also in lower expected heterozygosity values. Overall the highest assay efficiency index was observed for AFLPs (61.9) and the lowest for RFLPs (3.2). RAPDs and SSRs (5.8 and 4.4, respectively) were comparable to RFLPs. In particular, for AFLPs the high assay efficiency index is due to the simultaneous detection of several polymorphic bands in a multiplex amplification per single reaction.

Genetic similarity

A summary of the genetic similarity estimates, calculated for each marker system, between pairs of lines of the various heterotic groups is shown in Table 2. All marker systems indicated that lines of BSSS origin were more similar in comparison to inbred lines of other heterotic groups. The mean value of the GS estimate was, as expected, lower for BSSS × LSC crosses than within the BSSS and LSC groups themselves. This is consistent with the common practice in maize breeding of preferentially developing hybrids between heterotic groups because they are expected to perform better than those from crosses within heterotic groups. The similarity ranged from 0.92 within LSC types, using AFLPs, to 0.00 within BSSS × miscellaneous types, using SSRs. The estimates of GS follow the same pattern across marker systems, i.e. higher estimates of similarity within the BSSS types and lower estimates within the LSC × miscellaneous types. Overall SSRs revealed the lowest similarity values and AFLPs the highest.

The genetic similarity trees produced from each marker system are presented in Fig. 1. In these trees inbreds were ordered as expected, though with exceptions, into the major groups BSSS and LSC.

Table 1 Level of polymorphism and comparison of informativeness obtained with RFLP, RAPD, SSR and AFLP markers in 33 maize inbred lines

Parameters	Marker system			
	RFLP	RAPD	SSR	AFLP
Number of assay units	53 (probe/enzymes)	25 (primers)	27 (primer pairs)	6 (primer combination)
Number of polymorphic bands	253	90	183	232
Number of loci	53	90[a]	27	232[a]
Average number of alleles per locus	4.8	2.0	6.8	2.0
Expected heterozygosity	0.63	0.36	0.72	0.34
Effective number of alleles per locus	3.2	1.6	4.4	1.6
Assay efficiency index	3.2	5.8	4.4	61.9

[a] Theoretical maximum number of loci

Table 2 Mean, minimum and maximum of the Dice genetic similarity coefficient (GS) calculated from different molecular marker systems for various groups of maize inbred lines

Marker system		GS value						
		Within heterotic groups			Between heterotic groups			Between all lines ALL ($n = 528$)
		BSSS ($n = 78$)	LSC ($n = 78$)	MISC ($n = 21$)	BSSS × LSC ($n = 169$)	BSSS × MISC ($n = 91$)	LSC × MISC ($n = 91$)	
RFLP	Min.	0.28	0.25	0.23	0.18	0.20	0.18	0.18
	Max.	0.79	0.86	0.90	0.59	0.49	0.56	0.90
	Mean	0.48	0.40	0.41	0.36	0.36	0.35	0.37
RAPD	Min.	0.34	0.38	0.31	0.37	0.36	0.29	0.29
	Max.	0.90	0.80	0.89	0.76	0.65	0.68	0.91
	Mean	0.64	0.57	0.53	0.58	0.53	0.53	0.56
SSR	Min.	0.13	0.08	0.08	0.07	0.00	0.04	0.00
	Max.	0.84	0.88	0.82	0.52	0.52	0.40	0.88
	Mean	0.38	0.31	0.28	0.27	0.22	0.20	0.26
AFLP	Min.	0.45	0.43	0.43	0.36	0.43	0.39	0.36
	Max.	0.89	0.92	0.89	0.61	0.64	0.62	0.92
	Mean	0.62	0.59	0.55	0.48	0.52	0.51	0.53

Discrepancies in forming subgroups within the major groups were observed as well as in the clustering of inbred lines of miscellaneous origins. Considering the BSSS-related lines the topology of each tree is unique with some evident similarity: the clustering of B14, B37, and B73 is, for example, fully conserved. On the LSC side, clustering was consistently reported by all methods with the exception of Va22, a line derived from C103, indicating that all methods aggregated lines of different origin. The Oh43-related lines (Oh43 and A619) were positioned within the Lancaster group only by RAPDs and AFLPs, while SSRs and RFLPs clustered these with BSSS lines (although Oh43 is usually considered a Lancaster type). Similarly to the RAPD-based tree, the clustering based on AFLP data produced a tree with a relatively narrow range of similarity values between the more-related and the more-distant pairs of inbreds. In spite of this, all the main clusters were confirmed by AFLP data.

Four pairs of very similar inbreds (B14 A, Cm109; Lo932, Lo944; A619, Oh43; and H55, H96) were clustered together by all marker systems, while in one additional case similar lines (Lo916 and Lo999) were consistently grouped in AFLP, SSR and RAPD trees.

Comparison between marker systems

All the estimates of correlation coefficients (rs) among available co-ancestry coefficients (fs) and genetic similarity (GS) data were highly significant ($P < 0.01$). RAPDs showed the lowest correlation ($r = 0.40$) with f values, RFLPs and SSRs intermediate values ($r = 0.57$ and $r = 0.53$, respectively), while AFLPs showed the highest value ($r = 0.62$). The rs among similarity data were also significant. Correlation coefficients of RAPD marker data ($r = 0.51$, $r = 0.57$, and $r = 0.52$ with RFLP, SSR and AFLP, respectively) with those obtained using other marker systems were lower than those among similarity estimates based on AFLPs, RFLPs, and SSRs ($r = 0.70$, $r = 0.67$ and $r = 0.59$, respectively between AFLP and RFLP, AFLP and SSR, and RFLP and SSR). The extent to which similarities were correlated varied considerably across the whole data set. When the set of pairwise data (528) was divided into two groups (according to the arithmetic mean of the observed GS range based on RFLP data: "more similar" lines with GS > 0.37 and "less similar" lines with GS < 0.37), it became evident that the genetic similarities estimated by different marker systems were mainly correlated only among similar lines, while the relationships among dissimilar lines were low and not significant. The GS values plotted against the estimate of co-ancestry value based on pedigree data followed the same pattern.

The co-phenetic correlation coefficients provided for each marker system indicate the extent to which the clustering of genotypes depicted in the trees accurately represents the estimates of genetic similarity between inbreds obtained with that marker system. Overall the co-phenetic coefficients were medium to high, with the RFLP (0.84) and AFLP (0.83) data resulting in the highest correlation, SSR (0.80) showing an intermediate value, and the RAPD (0.72) assay producing the lowest correlation.

All methods could clearly distinguish all 33 inbred lines, although the SSR data provided the highest level of discrimination between any pair of inbreds. In general, the grouping agreed with the pedigree information of the lines, although some discrepancies were

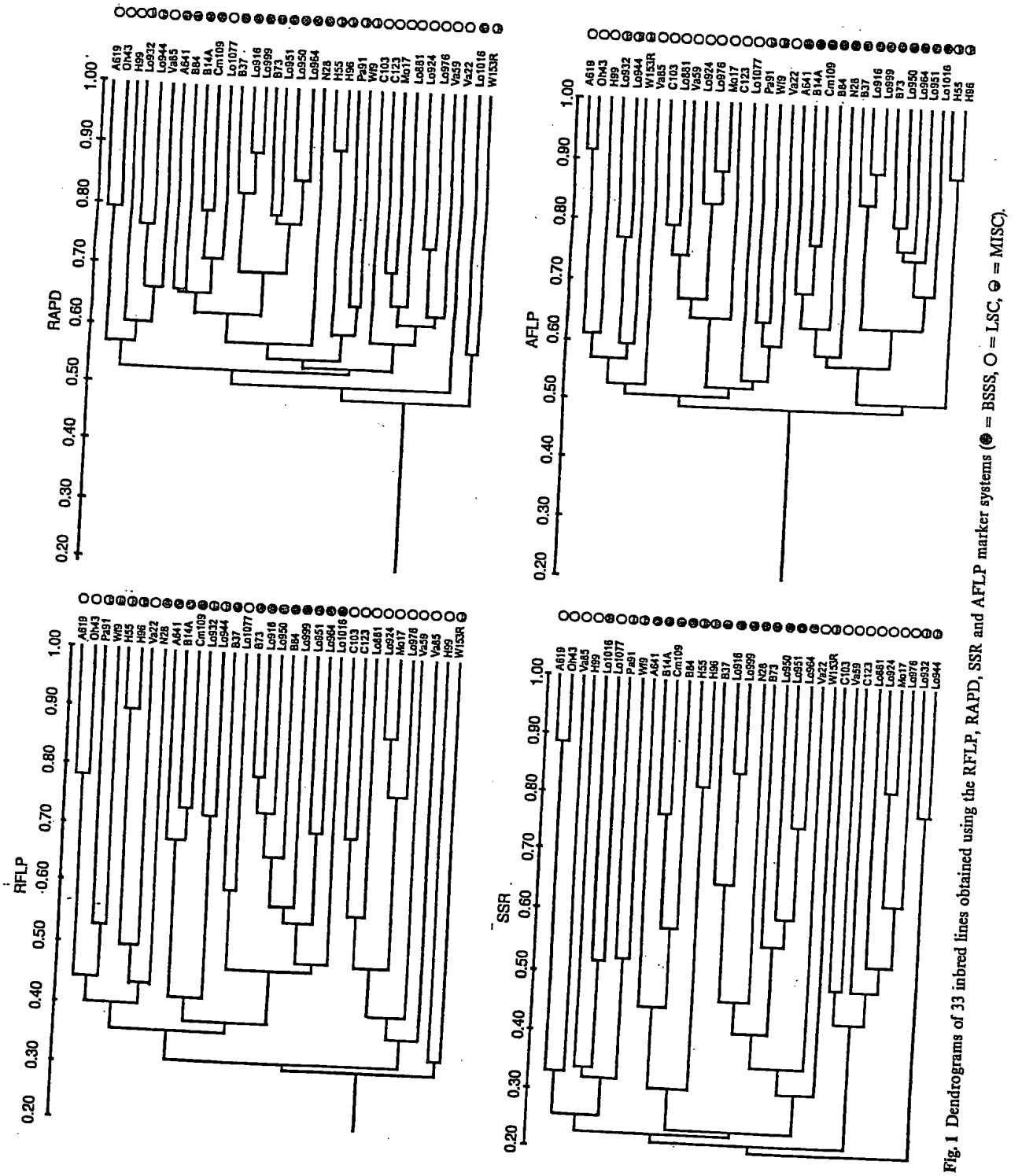

Fig. 1 Dendrograms of 33 inbred lines obtained using the RFLP, RAPD, SSR and AFLP marker systems (● = BSSS, ○ = LSC, ⊖ = MISC).

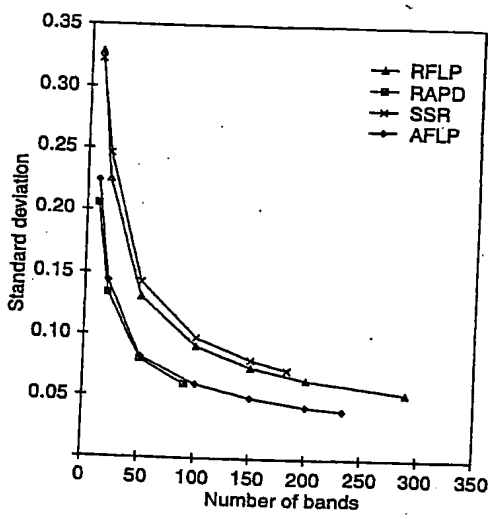

Fig. 2 Variation assessed by bootstrap sampling of genetic similarity between maize inbreds across different marker systems due to the different number of markers (bands)

observed. In particular, genetic similarities based on AFLP data had the highest correlation with pedigree data, while those based on RAPDs had the lowest one.

Bootstrap analysis

To determine the sampling variance of genetic similarities calculated from different molecular marker data sets, bootstrap analysis with a declining number of bands was performed. The relationships between number of bands and the sampling variance of the genetic similarity among all pairs of inbred lines for each method is presented in Fig. 2. The results indicated that above 150 bands there was a diminishing return in the precision gained by adding additional bands. As the number of bands moves below these thresholds the standard deviation begins to increase (and precision decreases) at a greater rate.

Discussion

In this paper we have shown that the number of alleles detectable in maize by SSRs is higher in comparison to other methods. This high level of polymorphism is to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz et al. 1986). It is also known that when SSRs have been compared to other marker systems they have revealed the highest level of polymorphism (Wu and Tanskley 1993; Morgante et al. 1994; Powell et al. 1996). The present data indicate that on average SSRs carry two-fold more information than AFLPs and RAPDs, and 40% more information than RFLPs, when the number of alleles per locus is the target.

In agreement with previous observations (Becker et al. 1995), the lowest degree of polymorphism was associated with AFLPs. Conversely, the information measured as the assay efficiency index, which correlates with the number of effective alleles identified per assay, was more than ten-fold higher for AFLPs compared to the other methods. These findings are in good agreement with previous germplasm analysis carried out in several crop species (Lu et al. 1996; Powell et al. 1996). It can be concluded that SSRs are capable of revealing the highest level of information per single marker and that AFLPs detect the highest number of polymorphisms in a single assay. This high assay efficiency index is a reflection of the efficiency of AFLPs to simultaneously analyse a large number of bands rather than the levels of polymorphism detected at each locus. The assay efficiency index for SSRs can, however, be considerably higher if multiplex PCR and gel-running procedures are adopted, where several microsatellites are simultaneously amplified and co-electrophoresed using multicolour fluorescent technologies (Lindqvist et al. 1996; Heyen et al. 1997). An additional advantage of SSRs over AFLPs is only relevant when mapping populations are derived from outcrossing heterozygous individuals, where the multi-allelism of SSR markers increases the number of informative genotypic classes in the progenies compared to the binary AFLP markers.

The results shown by genetic similarity trees indicate that, except for RAPDs, they are highly similar. In addition, trees from these molecular methods agree with the information obtained from pedigree data. Similarly, Powell et al. (1996) found the lowest correlations among RAPDs and other marker systems. In this respect, it has been shown that RAPD analysis, based on the use of random primers, is likely to suffer from a lack of reproducibility due to mismatch annealing (Neale and Harry 1994). In the trees obtained from cluster analysis, all lines with defined affiliation to one of the heterotic groups were assigned to their specific main clusters, in agreement with the available data for maize (Ajmone-Marsan et al. 1992; Livini et al. 1992; Mumm and Dudley 1994; Smith et al. 1997). A second observation is that, within the clusters, the grouping of more distantly related lines does not match precisely with the expectations based upon pedigree data. Differences among marker techniques in grouping genetically more distant lines have been previously reported (Powell et al. 1996). Other studies in *Brassica* and from pea accessions show that molecular marker-based similarities and trees were significantly correlated across a wide range of germplasms (Thormann et al. 1994; Lu et al. 1996). Many potential reasons for these discrepancies exist, including underlying assumptions in calculating pedigree data (Messmer et al. 1993), genome sampling

(Nei 1987), and the numbers of markers or probes employed (Tivang et al. 1994).

The number of loci required for a reliable estimate of genetic similarity has been shown to vary from 15 RFLP probes, giving 56 bands in *Brassica* sp. (dos Santos et al. 1994), to 100 RFLP clone-enzyme combinations (Messmer et al. 1993). Similarly, Tivang et al. (1994), investigating in maize the sampling variance of a RFLP data set in maize, found that the number of bands required for a CV of 10% was 388, 150, and 38 for closely, intermediately, and distantly related inbreds, respectively. Our results using the bootstrap procedure suggest that 150 bands are sufficient for reliable estimates of genetic similarity. Accordingly, the average number of assays that could have been used in this study to attain such a precision in the estimate were 30–40 clone-enzyme combinations for RFLPs, 40–50 primers for RAPDs, 20–30 primers for SSRs, and 4–5 enzyme combinations for AFLPs. Based on these estimates, the disagreement of the RAPD results in comparison to the other types of markers might be explained by the insufficient number of primers used.

In conclusion, the results of this study indicate that, with the exception of RAPDs, the other DNA markers provide consistent information for germplasm identification and pedigree validation. We have shown that SSR and AFLP profiling technologies can be good candidates to replace RFLP markers in genetic similarity estimates and variety description, and that they have comparable accuracy in grouping inbred lines selected by pedigree. They are generally much simpler to apply and more sensitive than the traditional morphological and biochemical methods or the RFLP-based fingerprinting techniques; yet they provide results correlated with those from RFLP analyses. A major advantage of the SSR and AFLP methods is that they can be automated. While SSRs, thanks to their multi-allelism and co-dominance, appear to be suited for the analysis of outcrossing heterozygous individuals, AFLPs, with their high multiplex ratio, offer a distinctive advantage when genome coverage is a major issue due to the presence of linkage disequilibrium, such as in inbred lines and breeding materials.

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Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs

Abstract RFLPs, AFLPs, RAPDs and SSRs were used to determine the genetic relationships among 18 cultivated barley accessions and the results compared to pedigree relationships where these were available. All of the approaches were able to uniquely fingerprint each of the accessions. The four assays differed in the amount of polymorphism detected. For example, all 13 SSR primers were polymorphic, with an average of 5.7 alleles per primer set, while nearly 54% of the fragments generated using AFLPs were monomorphic. The highest diversity index was observed for AFLPs (0.937) and the lowest for RFLP (0.322). Principal co-ordinate analysis (PCoA) clearly separated the spring types from the winter types using RFLP and AFLP data with the two-row winter types forming an intermediate group. Only a small group of spring types clustered together using SSR data with the two-row and six-row winter varieties more widely dispersed. Direct comparisons between genetic similarity (GS) estimates revealed by each of the assays were measured by a number of approaches. Spearman rank correlation ranked over 70% of the pairwise comparisons between AFLPs and RFLPs in the same order. SSRs had the lowest values when compared to the other three assays. These results are discussed in terms of the choice of appropriate technology for different aspects of germplasm evaluation.

Key words Barley · Genetic relationships · Molecular analysis · RFLP · AFLP · RAPD · SSR

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Introduction

As one of the first crop plants to be domesticated, barley (*Hordeum vulgare* L.) remains one of the most important crops today. Ranking fourth in world acreage, barley is used for human consumption, as a fodder crop and as a raw material for brewing beer and whisky (Brown 1992). It belongs to the genus *Hordeum*, which comprises over 32 species, including diploid and polyploid, perennial and annual types, which are spread throughout the world. The genus can be divided into three groups of varying importance to cultivated barley improvement; the primary genepool (*H. vulgare* spp. *vulgare* and *H. vulgare* spp. *spontaneum*), the secondary genepool (*H. bulbosum*) and the tertiary genepool (all other *Hordeum* species). Presently more than 250,000 *Hordeum* accessions are held in genebanks throughout the world, and the number is increasing (IBPGR 1992). With the growth of the germplasm collection a need for procedures which will allow their more effective use is required. The 'Core Collection' concept, is one such method, which should provide users with a limited set of genetically distinct and representative accessions (Brown 1989). Recently this concept has been applied to barley, and the Barley Core Collection (BCC), consisting of a limited sample of accessions considered to represent the spectrum of genetic diversity available in the genus, was established (Hintum 1992).

In such collections, morphological data are the principle descriptors which have been used to detail the accessions held. With the development of molecular markers and their many perceived advantages, it is crucial that these techniques are applied to assess genetic diversity in germplasm collections in order to supplement and refine the morphological-based classification. However, in recent years, the number of molecular assays available for application in this area has increased dramatically, with each method differing in principle, in application, in the type and amount of polymorphism detected and in cost and time

requirements. The approaches include restriction fragment length polymorphism (RFLPs; Botstein et al. 1980), random amplified polymorphic DNA (RAPDs; Williams et al. 1990), simple sequence repeat polymorphisms or microsatellites (SSRs; Tautz 1989) and Amplified Fragment Length Polymorphism (AFLPs; Zabeau and Vos 1993).

Faced with this wealth of marker technology, it is appropriate to determine if the same patterns of variability are revealed by each and whether the observed molecular diversity reflects either co-ancestry or morphological classification. To address this we have evaluated and compared similarity measures obtained from the four above systems on a set of accessions which are representative of cultivated European barley germplasm. This has allowed us to compare the results obtained from molecular analysis with each other and with pedigree information. The results are discussed in relation to the overall genetic diversity observed and the features of the individual assays.

Materials and methods

Plant material and DNA isolation

Eighteen accessions (Table 1), representing the majority of ancestors European cultivated barley, were selected for this study. Total genomic DNA was isolated from fresh leaf material by a modification of the method described by Saghai-Maroof et al. (1984).

Marker analysis

RAPD

RAPD amplifications were performed as described by Barua et al. (1993). Fragments were separated on 1.5% agarose gels, stained with ethidium bromide, visualised with ultraviolet light and photographed. The presence or absence of polymorphic bands were scored. Twenty primers, which were polymorphic between the parents of a spring × spring cross ('Blenheim' and E224/3), were used in this study.

RFLP

RFLP profiles were detected according to the protocol described by Graner et al. (1991). DNA was digested with three restriction enzymes (*Bam*HI, *Eco*RI and *Hin*dIII), and restriction fragments were detected using 48 single-copy DNA clones selected from previous mapping experiments to give good genome coverage and levels of polymorphism (Graner et al. 1991). RFLP patterns were scored as presence or absence of bands.

SSR

Two sources of simple sequence repeats were used in this study: database-derived repeats and repeats derived from an enriched genomic library. The 6 database-derived SSRs are described in a recent publication by Becker and Heun (1995). The 7 library-derived SSRs are described by Macaulay et al. (in preparation). SSR assays were performed as described by Morgante et al. (1994). Allele lengths were determined by comparing the most intense band with an M13 DNA sequence marker.

Table 1 Pedigree information and country of origin of 18 barley accessions used in molecular analysis

Cultivar	Pedigree	Origin[a]
Spring type, two-rowed:		
Aramir	Volla × Emir	NL
Beka	Bethge XIII × Kniefel	F
Golden Promise	X-ray mutant from Maythorpe (Irish Goldthorpe × Maja)	GB
Grit	Langenstein-Nungesser (5547/67 × 46459/68) 480/68 or Hadml. 554-Emir-11191-Union-46495-Diamant 14008	D
Hora	Sultan × (Weihenstephaner 1206 Nacktgerste × Volla)	D
Krona	Complex cross including Triumph	D
Triumph	(Hadm.24566 × Diamant × 1402964/6) × ((Alsa × Abyssinian) × St. × Union)	D
Union	(Weihenstephaner Mehtauresistente II × Donaria) × Firlbecks III	D
Volga	Complex cross with eight varieties	F
Winter type, two-rowed:		
Igri	(Malta × Carlsberg 1427) × Ingrid	D
Marinka	(Alpha × SVP 674) × Malta	NL
Romanze	Weihenstephan 4622/73 × (Malta × Sonja)	D
Sonja	Tria × Malta	D
Winter type, six-rowed:		
Borwinia	Vogelsanger Gold × St. 7246	D
Express	Robur × Athene	D
Franka	(Vogelsanger Gold × Senta) × (Dura × Dea) × Vogelsanger Gold	D
Gaulois	Gerbel × Athene	F
Rondo	Tanaroo × Sisfor L. 90	I

[a] NL, The Netherlands; F, France; GB, Great Britain; D, Germany; I, Italy

AFLP

AFLP analysis was essentially as described by Vos et al. (1995). Briefly, 500 ng of genomic DNA was digested with *Eco*RI and *Mse*I and double-stranded adaptors ligated to the fragment ends. This was followed by a pre-amplification step using non-selective primers. Selective amplifications were performed on the pre-amplified fragment mixture using a total of six primer combinations. Only the *Eco*RI primer was radiolabelled with γ-[^{33}P] ATP (ICN), and all primers had three selective nucleotides. Amplification products were separated by denaturing 6% polyacrylamide gel electrophoresis (PAGE), visualised by autoradiography and manually scored for the presence or absence of bands.

All of the primer names and sequences used are available on request from the authors.

Data analysis

Diversity values were calculated for each locus as $(1 - \Sigma\, Pi^2$, where Pi is the phenotypic frequency for each assay unit (RFLPs-probe/enzyme combinations; RAPDs-primers; SSRs-primer pairs; AFLPs-primer combinations). Genetic similarities (GS) were calculated using the GENSTAT Version 531 software package according to Nei and Li's (1979) estimate of similarity. Similarities were expressed using the group average agglomerative clustering function of GENSTAT to generate principal co-ordinate plots (Kempton and McNicol 1990). Correlations between assays were calculated using [Procrustes rotational analysis (PR) on the principal co-ordinate data] Spearman rank correlation (SRC) and linear regression of the GS values.

Results

Fingerprinting

All of the molecular approaches used in this study were able to uniquely fingerprint each of the 18 cultivated barley accessions. The total number of assay units varied for each marker system from only 6 primer combinations for AFLPs to 144 probe/enzyme combinations for RFLPs (Table 2). Similarly, the number of bands scored ranged from 70 for SSRs to 299 for RFLPs. The percentage of polymorphic bands for each assay did not correlate to the total number of bands. For example, only 70 bands were scored for SSRs, which was the lowest number, but all 70 were polymorphic. In contrast, 297 AFLP bands were scored, and only 46.8% of those were polymorphic. RFLPs and RAPDs were intermediate with 83.2% and 66.3%, respectively, of all bands scored being polymorphic. There was wide variation in the average number of genotypes revealed by each marker system (Fig. 1). With RFLPs, for each probe/enzyme combination, an average of 2.37 genotypic classes could be distinguished. With AFLPs this figure increased to 17.2 as nearly all primer combinations were able to discriminate between the 18 accessions used. This is further reflected in the diversity index measures. Overall the highest diversity index was observed for AFLPs (0.937), and the lowest for RFLPs (0.322). RAPDs and SSRs were intermediate (0.521 and 0.566, respectively).

Genetic similarity

The cultivated barley genepool can be divided into spring and winter types. The winter barleys are mainly used for fodder and can be further divided into two- and six-rowed types. The spring barleys are mainly used for malting. The maximum, minimum and mean similarity estimates between the spring barleys and two-row and six-row winter barleys for each assay system are shown Table 3. The similarities ranged from 0.97 within spring types using AFLPs to 0.45 within six-row winter types using SSRs. Between assay systems the estimates of similarity followed the same pattern, i.e. higher estimates of similarity within the spring types (means: RFLPs = 0.843, AFLPs = 0.924, SSRs = 0.829) and lower estimates within the six-row winter types (means: RFLPs = 0.70, AFLPs = 0.877, SSRs = 0.657). Estimates with two-row winter types were intermediate. The situation with RAPDs was different, with spring and six-row winter types exhibiting equivalent mean similarities (0.879 and 0.897, respectively). Overall, SSRs revealed the lowest similarity values (0.93–0.45) and AFLPs the highest (0.97–0.81).

Some accessions can be traced to common ancestors. For example, Grit and Triumph have Union in their pedigrees and Krona has Triumph. Our expectation would therefore be that these 4 accessions should be closely related. Table 4 shows the genetic similarity

Table 2 Analysis of the RFLP-, RAPD-, SSRs- and AFLP-generated banding patterns

Marker	Number of assay units	Total no of bands	Number of polymorphic bands (%)	Number of bands per assay unit	Number of phenotypes per assay unit	Diversity index
RFLPs	114 (42 probes, 3 enzymes)	299	249 (83.2%)	2.62	2.37	0.322
RAPDs	22 (primers)	107	71 (66.3%)	4.86	3.41	0.521
SSRs	13 (primer pairs)	70	70 (100%)	5.38	5.38	0.566
AFLPs	6 (primer combinations)	297	139 (46.8%)	49.5	17.2	0.937

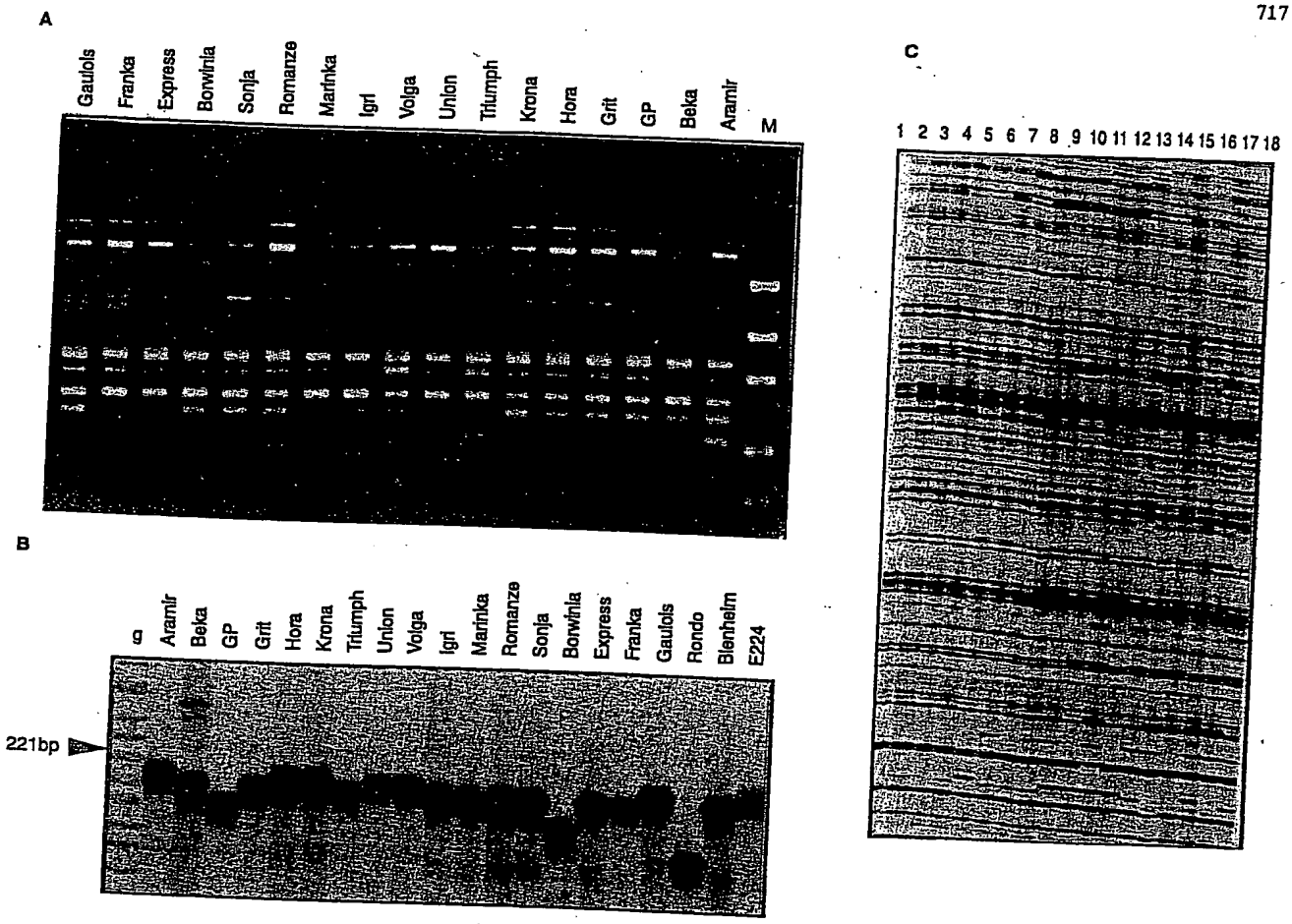

Fig. 1A–C An example of the different information content observed with RAPDS (A), SSRs (B) and AFLPs (C)

values for the comparisons of these 4 accessions with each of the molecular assays. With RFLPs, AFLPs and SSRs the genetic similarity values were higher than the mean values for all the spring types, and the highest similarity was between Triumph and Grit (RFLPs 0.93, AFLPs 0.97, SSRs 0.97). From the pedigree information in Table 1, Triumph and Grit share a number of parental lines including Union, Diamont and Hadm. With RAPDs, the genetic similarity values were less than the average, although the Triumph and Grit comparison was again the highest.

The genetic similarity values for the two-row winter varieties were intermediate between the spring and six-row winter types for RFLPs, AFLPs and SSRs. Sonja and Romanze were more similar than the other two-row winter types with values of 0.84 (SSRs), 0.93 (AFLPs), 0.89 (RFLPs) and 0.94 (RAPDs). This was not

Table 3 Maximum, minimum and mean genetic similarity estimates calculated from RFLP, RAPD, SSRs and AFLP data for winter and spring types

	RFLPs			RAPDs			AFLPs			SSRs			Parentage		
	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
Spring	93.0	76.0	84.3	95.0	84.0	87.9	97.0	88.0	92.4	93.0	66.0	82.9	0.330	0.020	0.133
Two-row winter	89.0	81.0	83.8	95.0	86.0	91.7	93.0	98.0	91.0	84.0	56.0	71.3	0.290	0.100	0.212
Six-row winter	85.0	60.0	70.0	95.0	88.0	89.7	91.0	81.0	87.7	90.0	45.0	65.7	0.350	0.000	0.111

Table 4 Genetic similarity values for the comparisons of 4 spring accessions with each of the molecular assays

	Grit	Krona	Triumph	Union	Maximum	Minimum	Mean
RAPDs:							
Grit	100.0						
Krona	88.0	100.0					
Triumph	90.0	85.0	100.0				
Union	88.0	88.0	84.0	100.0	95.0	84.0	87.9
RFLPs:							
Grit	100.0						
Krona	85.0	100.0					
Triumph	93.0	88.0	100.0				
Union	87.0	87.0	86.0	100.0	93.0	76.0	84.3
AFLPs:							
Grit	100.0						
Krona	91.0	100.0					
Triumph	97.0	92.0	100.0				
Union	93.0	94.0	94.0	100.0	97.0	88.0	92.4
SSRs:							
Grit	100.0						
Krona	84.0	100.0					
Triumph	97.0	87.0	100.0				
Union	69.0	70.0	94.0	100.0	97.0	66.0	82.9

unexpected as the co-efficient of parentage values were also the highest (0.290 for Sonja × Romanze compared to the mean for two-row winter type of 0.212). Both Sonja and Romanze are related through Malta, and Romanze has Sonja in its pedigree. The lowest genetic similarity values were observed for comparisons with Rondo.

Genetic relatedness

Associations among the 18 accessions were revealed by principal co-ordinate analysis (PCoA) (Fig. 2). The PCoA for the combined data (775 bands) clearly separated the winter from the spring accessions. Among the winter types, the two-rowed and six-rowed varieties formed two distinct groups, with the two-rowed types forming an intermediate group between the spring and six-rowed winter types. In the PCoAs generated by RFLP (299 bands) and AFLP (297 bands) data, a similar arrangement was observed. From the RAPD data, three distinct groups were again observed, although the spring types were more dispersed. Only a small group of spring types clustered together using SSR data, and two-row and six-row winter types were again more dispersed. On all of the PCoAs, Rondo appears in a remote position. In addition, 'Volga', a spring variety, was positioned between the rest of the spring and the two-rowed winter types.

Comparison between assays

To compare the results obtained with the four techniques, we tested correlations using Procrustes rotation (PR), linear regression of the pairwise GS values (LR) and Spearman rank correlation (SRC). The results for SRC (which compares how each system ranks pairwise similarities) are shown in Table 5. Comparisons using PR and LR showed the same general trends although the overall correlations were lower. Over 70% of the pairs of genotypes were ranked in the same order with RFLPs and AFLPs. This correlation is reduced to 10.9% when comparing RAPDs with AFLPs. SSRs were intermediate with over 50% of the genotypes ranking in the same order as that obtained with AFLPs and RFLPs.

Discussion

Given the proliferation of genetic markers, comparisons between techniques are inevitable. However, there is a need for such comparisons in order to decide on which technique is best suited to the issues being examined. In this study, three of the newer polymerase chain reaction (PCR)-based systems (RAPDs, SSRs and AFLPs) developed during the last 5 years have been compared with the well established RFLP system that was developed over 15 years ago. Each technique not only differs in principal, but also in the type and amount of polymorphism detected. The levels of polymorphism between the four techniques varied widely, ranging from a maximum of 100% (SSRs) to only 48.6% (AFLPs). Similar results were observed when Rus-Kortekaas et al. (1994) directly compared SSRs with RAPDs in tomato where the level of polymorphism was 40% with RAPDs compared to 100% with

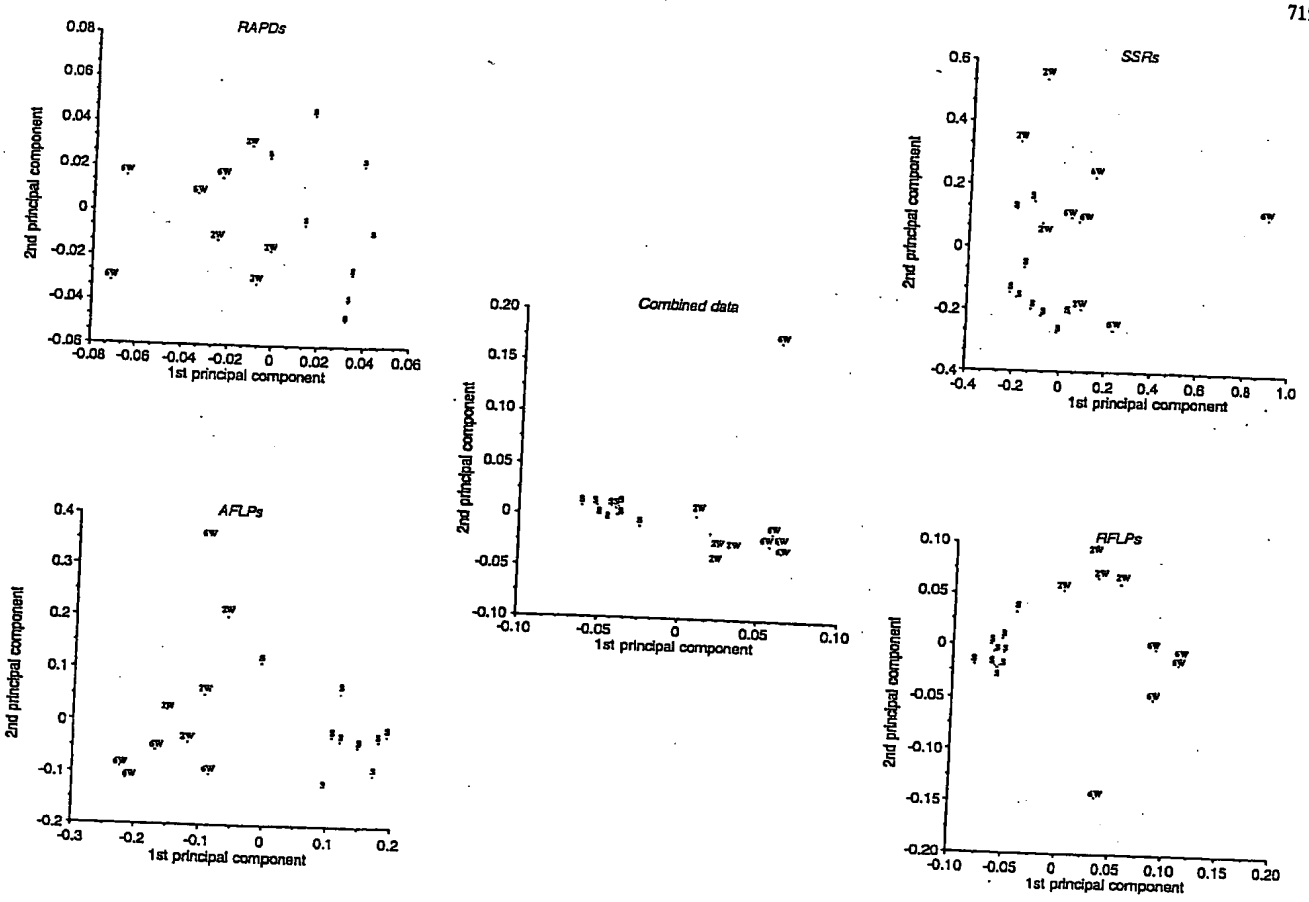

Fig. 2 Associations among the springs and winters cultivars revealed by principal co-ordinate analysis for each molecular assay

Table 5 Correlations obtained using RFLPs, RAPDs, AFLPs and SSRs based on Spearman's rank correlation and Procrustes rotation

SSR	1.000			
AFLP[a]	0.515	1.000		
RAPD[a]	0.235	0.109	1.000	
RFLP	0.505	0.708	0.201	1.000
	SSR	AFLP	RAPD	RFLP

[a] Rondo omitted from dataset for RAPD comparisons

SSRs. Indeed, whenever SSRs have been compared to other systems, they have always revealed the highest levels of polymorphism (Rus-Kortekaas et al. 1994; Salimath et al. 1995; Saghai Maroof et al. 1994; Powell et al. 1996; Maughan et al. 1995; Morgante et al. 1994; Wu and Tanksley 1993). The level of polymorphism detected using RFLPs in this study was higher (83.2%) than that observed in previous studies on barley using a similar selection of genotypes (46%) (Melchinger et al. 1994). This is probably due to pre-selection of polymorphic RFLP probes. The lowest level of polymorphism was associated with AFLPs. Becker et al. (1995) also observed that levels of polymorphism revealed by AFLPs were lower than by RFLPs. However, although AFLPs do not offer the highest level of polymorphism, they are the most efficient because they have the capacity to reveal many polymorphic bands in a single lane. The average number of bands per lane or per PCR for AFLPs was 49.5, compared to 1.0 band per lane or PCR for SSRs. Thus, when the overall diversity indices of the four techniques were compared, AFLP was the highest (0.937). Powell et al. (1996) introduced the concept of Marker Index as an overall measure of marker efficiency, and they demonstrated that, in *Glycine*, AFLPs had the highest Marker Index compared to other available marker systems. The high Marker Index or diversity index is a reflection of the efficiency of AFLPs to simultaneously analyse a large number of bands rather than the levels of polymorphism detected.

Barley germplasm can be divided into two genepools, winter and spring, based on morphology distinctions. Melchinger et al. (1994) using RFLPs observed a clear separation between the spring and winter

types. In this study, similar results were observed using RFLPs, AFLPs, RAPDs and SSRs. Furthermore, Melchinger et al. (1994) noted that sub-groups were also apparent for accessions with similar pedigrees, such as the compact grouping of two-row winter types intermediate between the spring and six-row winter types. With the exception of the SSR data, the two-row winter types form a sub-group between the six-row winter and the spring types for RFLP, AFLPs and RAPDs. With the SSR data there is a clear separation between the spring and winter types, but not within the winter types. This is not unexpected considering the low level of band sharing between accessions; even within groups the estimates of genetic similarity were much lower than any of the other assays.

Several previous studies have compared the use of RFLPs and RAPDs to examine genetic relatedness (Hallden et al. 1994; Thormann et al. 1994; Liu and Furnier 1993; dos Santos et al. 1994), and most of these show that RAPDs and RFLPs detect very similar relationships among the same group of accessions. Recently, other reports have compared RAPDs or RFLPs and SSRs on the same set of genotypes (Rus-Kortekaas et al. 1994; Wu and Tanksley 1993; Salimath et al. 1995; Maughan et al. 1995). Rus-Kortekaas et al. (1994) observed a lower percentage of band sharing in tomato accessions with SSRs compared to RAPDs and suggested that higher band sharing would make RAPDs more suitable for genetic relatedness studies. The results in this study would support the finding that SSRs may not be particularly well suited for pedigree relationship studies, although only a small number of SSRs were used.

Knowledge of genetic variation and the genetic relationship between genotypes is an important consideration for efficient rationalisation and utilisation of germplasm resources. Furthermore, it is important for the optimal design of plant breeding programmes, influencing the choice of genotypes to cross for the development of new populations. In barley, breeders have made crosses between highly selected genotypes with the result that the number of genotypes within the breeding genepool is very small. According to Graner et al. (1994) better knowledge and measures of genetic similarity of accessions could help to maintain genetic diversity. In the past, indirect estimates of similarity based on pedigree information have been widely used in many species including barley. Such estimates may not always reflect the true relationships between accessions (Graner et al. 1994). In this study we have used molecular markers to determine direct measures of genetic similarity between individuals. The estimates varied from 0.97 (AFLPs) to 0.45 (SSRs). Melchinger et al. (1994) reported GS values of 0.79 for unrelated barley pairs, based on RFLPs. The RFLP results reported in this paper were similar to these. Also, Tinker et al. (1993) observed GS values in a set of 27 North American barley cultivars using RAPDs which were similar to those found here (0.84–0.95). The values of GS based on SSRs in this present study are much lower than those based on RFLPs, AFLPs and RAPDs. Rus-Kortekaas et al. (1994) reported that the percentage of band sharing between tomato cultivars using SSRs was only 50.8% compared with 82.7% for RAPDs. Plaschke et al. (1995) observed even lower (0.31) estimates of genetic similarity when employing SSRs to examine wheat accessions and suggested that these low values are a reflection of the high information content provided by SSRs.

Although we have shown that molecular approaches can be used to group barley cultivars into morphologically distinct groups, and also further into sub-groups which have a similar genetic background, we have not addressed the issue of concordance of molecular-based estimates of GS and co-ancestry. Graner et al. (1994) compared RFLP-based estimates of GS with co-ancestry for a set of 48 cultivars. A very weak correlation was reported; $r_s = 0.21$ for winter and $r_s = 0.42$ for spring types. Similarly, using protein-based gliadin markers Cox et al. (1985) observed a correlation of $r_s = 0.27$. Both Graner et al. (1994) and Cox et al. (1985) agree that perhaps the reason for these poor correlations may be the high background similarity found for unrelated accessions using molecular markers. When related cultivars were used to investigate correlations between RAPD-based estimates of GS and co-ancestry a moderate correlation of $r_s = 0.61$ was observed between both measures (Tinker et al. 1993). Plaschke et al. (1995) observed similar results in wheat using SSR-based GS estimates and pedigree measures ($r_s = 0.55$). Although we have only a limited set of co-ancestry measures for the accessions studied here, several conclusions can be drawn from the correlations between molecular estimates of GS and the co-efficient of parentage. For example, the co-efficient of parentage for Rondo was 0 for all of the pairwise comparisons, and with all molecular measures Rondo had the lowest GS value. The low-to-moderate correlations between molecular measures of GS and pedigree estimates have led to the conclusion that pedigree information may not be as useful for certain applications for which they have been used in the past (Graner et al. 1994; Plaschke et al. 1995). In any case, molecular-based estimates of GS will provide more information than is available from pedigree information.

Having established that molecular-based estimates of GS will allow plant breeders to make informed decisions regarding the choice of genotypes to cross, we must ask the question as to which assay is most appropriate? Several studies have been described which address this question using isozymes, RFLPs and RAPDs (dos Santos et al. 1994; Thormann et al. 1994; Heun et al. 1994; Hallden et al. 1994). Heun et al. (1994) found that the correlation between RAPDs and isozymes among *Avena sterilis* accessions were moderately low

($r_s = 0.36$), although the overall representation of genetic relatedness was in considerable agreement. Beer et al. (1994) assessed genetic variation among *Avena sterilis* using morphological markers, isozymes and RFLPs and found a similarly low correlation ($r_s = 0.27$). A very different situation was observed among *Brassica* species. Thormann et al. (1994) reported correlations of $r_s = 0.969$ between RFLPs and RAPDs for a group of 18 accessions from different *Brassica* species. Dos Santos et al. (1994) also observed a significantly high correlation between RFLPs and RAPDs ($r_s = 0.745$) using genotypes within *Brassica oleracea*, although they did observe differences between the RFLP and RAPD dendrograms. When Spearman rank correlation was used, AFLPs and RFLPs ranked over 70% of the pairwise comparisons in the same order. This may well be because both techniques are based on restriction site changes, the major difference is that PCR is used in AFLPs rather than Southern analysis in RFLPs. In contrast SSRs and RAPDs have the lowest values when compared to the other assays. The low correlations observed with RAPDs could be a reflection of the choice of primers which we have previously used in the construction of a linkage map using a population derived from two related spring varieties. This may well have resulted in biased estimates of GS, which in turn has affected the ranking order of genotypes. For example, the lowest GS was between two spring types (Volga and Beka; GS = 0.84), whereas the lowest GS estimates for the other assays were between two winter types (even when Rondo was removed from all the data sets this still holds true).

The lack of correlation between SSRs and the other assays may not be fully unexpected, considering the high levels of polymorphism between pairwise comparisons. Powell et al. (1996) reported that SSRs were well-correlated with AFLPs and RFLPs at the interspecies level, however at the intraspecies level the correlation disappeared, emphasising the uniqueness of the SSR assay. Thus, while SSR analysis appeared to be the most polymorphic assay system, it did not seem to be particularly useful for assessing genetic relationships among cultivars. RFLPs were particularly valuable for assessing genetic relationships, but required several probe and enzyme combinations to discriminate between accessions. Both RFLPs and SSRs require an initial investment in terms of probe or sequence information, and according to Vos et al. (1995) the ideal fingerprinting assay should require no prior sequence knowledge. While only AFLPs and RAPDs meet these requirements, the lack of comparative information at each assayed locus (due to dominance) precludes an accurate assessment of true genetic relationships.

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